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The hypothesis of this proposal is that breast cancer cells are capable of breaking down the extracellular matrix barrier and this plays an important role in breast cancer invasion and metastasis. This study is based on our previous observation that breast cancer tissue expresses a meprin-like protein, which can be identified by immunostaining of breast cancer tissue using anti-meprin antibody. During the first year, our research was focused on the expression of meprin alpha in bacteria and raising antibody to the recombinant protein in order to have a tool for the identification of the meprin-like protein in tissues and extracted proteins. In addition, we performed immunohistology studies of human breast tumor and kidney tumor tissues, and produced data on experimental tumor growth with and without inhibition of meprin.

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Introduction

The hypothesis of this proposal is that breast cancer cells are capable of breaking down the extracellular matrix barrier and this plays an important role in breast cancer invasion and metastasis. This study is based on our previous observation that breast cancer tissue expresses a meprin-like protein, which can be identified by immunostaining of breast cancer tissue using anti-meprin antibody.

Body

During the first year, our research was focused on the expression of meprin alpha in bacteria and raising antibody to the recombinant protein in order to have a tool for the identification of the meprin-like protein in tissues and extracted proteins. In addition, we performed immunohistology studies of human breast tumor and kidney tumor tissues, and produced data on experimental tumor growth with and without inhibition of meprin.

Expression of meprin in bacteria and purification of the recombinant protein

Rat meprin alpha cDNA coding sequence was amplified along with initiating and stop codons using primers 5'...AACAATGCTGTGGACACTACCTGT (sense) and 5'...GAGGTTTGGGGCTTTGGTTTTGGA (antisense), and cloned into pGEM-T vector (Promega). The sequence was compared with meprin alpha (Genbank # S43408), and the homology was confirmed. The sequence was excited and re-cloned into pMal-c2 bacterial expression vector (New England Biolabs, Beverly, MA) between EcoR1 and HindIII restriction sites. The construct resulted in a fusion cDNA with maltose-binding protein (MPB) at the 5' end was transfected into E.coli BL21DE3pLyS competent cells (Novagen). Positive clones were selected using PCR of ampicillin-resistant colonies.

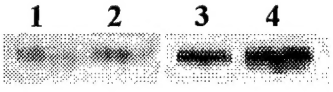

Figure 1. Western blotting of meprin in total rat kidney extracts using polyclonal antibody raised to purified meprin (dilution 1:100; 1 & 2, 50 and 100 ug protein, respectively) or recombinant fusion meprin-MPB protein (dilution 1:12,000; 3 & 4, 50 and 100 ug protein, respectively).

The effect of temperature, time of induction (IPTG) and the presence of protease inhibitors to the protein production in the cytoplasm were tested. The fusion meprin-MPB protein (expected size 115 kDa) was extracted from the cytoplasm, purified using affinity chromatography on amylose resin, and quantified. We observed that the temperature of 30°C, the IPTG induction for 2 hours and the use of protease inhibitors resulted increased cytoplasmic meprin alpha production. The

extraction from inclusion bodies showed increased yield, however the fusion protein was denatured during purification because of treatments with high osmolarity, detergents, and urea [FEBS, 307:375-378, 1992]. Because the proper folding of the protein after purification from inclusion bodies could not be achieved, this approach was not used for obtaining active enzyme.

Raising and characterization of anti-meprin antibody

In attempt to cleave MPB from meprin alpha, digestion with Factor Xa (New England Biolabs) was used as suggested by the manufacturer. We found that Factor Xa degrades more meprin alpha itself rather than cleaves it out of the fusion protein. Thus, for immunization of rabbits, we used fusion protein without digestion. The protein for immunization was prepared from E.coli soluble fraction, purified by affinity chromatography, and dialyzed. Female rabbits were injected intradermally with 100 μg protein. Two 400 μg boosters in one and two months after the first injection was used to induce the antibody production. Blood serum was collected before the first injection and at every step of immunization. They were analyzed by Western blot against recombinant protein and protein extracts from rat kidney. The obtained antiserum was compared to the antibody produced previously in this laboratory from meprin alpha purified from rat kidneys. We observed that the antiserum presented the same pattern with the previously raised anti-meprin antibody, and therefore can be used for immunological identification of the meprin-like protein (Fig.1).

Western blotting and immunohistology of tumor tissues

Western blotting analysis using the anti-meprin antibody stained a band of approximately 65 kDa in breast tumor, normal human kidney and human kidney homogenates. We stained various breast tumors obtained from VA Hospital Pathology Department (S98-1274, S98-1279) and from Birmingham tumor tissue repository (96-04co25F, 96-03OCo92R, 96-03-co93R, 0013679A (all kidney tumors), 134741, 0013716D, 0013717D, 0013717Q (all breast tumors). Although the staining was mostly negative, all the tissues were positive for 65 kDa protein on Western analysis.

Table 1. Size of tumor xenografts (dimentions, cm) developed in mice treated with actinonin.

Dorsal Flank	
Control:	**With Actinonin:**
1. 1.20 ± 0.85	1. 1.10 ± 0.75
2. 0.95 ± 0.60	2. 0.60 ± 0.50
3. 1.30 ± 0.80	3. 0.75 ± 0.40
4. 1.20 ± 0.80	4. 0.50 ± 0.40
Mammary Pad	
Control:	**With Actinonin:**
1. 1.15 ± 0.80	1. 1.40 ± 0.90
2. 0.95 ± 0.60	2. 1.30 ± 1. 10
3. 1.35 ± 0.95	3. no tumor
4. 1.20 ± 0.85	4. no tumor

Inhibition of tumor development by actinonin

Actinonin is the inhibitor of meprin. We hypothesized that injection of actinonin prior to the injection of breast cancer cells may suppress the tumor xenograft development. MCF-7 cell line was used to induce tumors in mice. The tumors were developed in two positions, dorsal flank and mammary gland. The actinonin was injected before the injection of breast cancer cells and one injection/ day day for 3 days post-

tumor cell injections. Our data presented in Table 1 provide preliminary evidence that actinonin inhibits xenograft development in mice. These data suggest that tumor development is dependent on the meprin or meprin-like proteinase activity in the tissue.

Key Research Accomplishments

- meprin alpha was expressed in bacteria and purified;
- polyclonal antibody raised to meprin alpha;
- performed immunostaining of breast tumor tissues with anti-meprin;
- antigen reacting with anti-meprin identified in tumor tissues using Werstern blotting;
- actinonin, inhibitor of meprin, was shown to inhibit development of breask tumors in mice.

Reportable Outcomes
N/A

Conclusions
Our data provide preliminary evidence that actinonin inhibits xenograft development in mice. These data suggest that tumor development is dependent on the meprin or meprin-like proteinase activity in the tissue. Developed polyclonal antibody to reconbinant meprin can be used for identification of meprin-like protein in breast tumor tissues.

References
Are included in the body

Appendices
N/A